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Standard and Fpg-modified comet assay in kidney cells of ochratoxin A- and fumonisin B_1-treated rats

Ana-Marija Domijan [a,*], Davor Želježić [b], Nevenka Kopjar [b], Maja Peraica [a]

[a] *Unit of Toxicology, Institute for Medical Research and Occupational Health, Ksaverska c. 2, 10000 Zagreb, Croatia*
[b] *Mutagenesis Unit, Institute for Medical Research and Occupational Health, Ksaverska c. 2, 10000 Zagreb, Croatia*

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Abstract

The effect of ochratoxin A (OTA), fumonisin B_1 (FB_1), and their combinations on DNA damage was studied using the standard alkaline comet assay and the Fpg-modified comet assay. Rats were orally receiving OTA (5 ng/kg b.w., 0.05 mg/kg b.w., and 0.5 mg/kg b.w., respectively) for 15 days, FB_1 (200 ng/kg b.w., 0.05 mg/kg b.w., and 0.5 mg/kg b.w., respectively) for 5 days, and the combinations of two lower OTA and FB_1 doses. The tail length, tail intensity, and Olive tail moment (OTM) obtained with the standard comet assay and Fpg-modified comet assay were significantly higher in treated animals than in controls, even at the lowest dose of OTA or FB_1 ($p < 0.01$). The Fpg-modified comet assay showed significantly greater tail length, tail intensity, and OTM in all treated animal than did the standard comet assay ($p < 0.05$), which suggests that oxidative stress is likely to be responsible for DNA damage. DNA damage detected by the standard comet assay at all OTA or FB_1 doses indicates that some other mechanism is also involved. Combined OTA + FB_1 treatment measured either by the standard comet or the Fpg-modified comet assay showed a synergistic increase in the tail intensity and OTM in kidney cells, even at doses that correspond to the daily human exposure in Europe.

Keywords: Single-cell gel electrophoresis; Free radicals; Fumonisin B_1; Kidney; Ochratoxin A; Oxidative stress; 8-OxodG

1. Introduction

Ochratoxin A (OTA) and fumonisin B_1 (FB_1) are nephrotoxic mycotoxins that contaminate various commodities, and humans are constantly exposed to low levels of these mycotoxins. Both mycotoxins are carcinogenic in experimental animals, but the mechanisms of their carcinogenicity are not known (NTP, 1989, 2001). However, in bacteria and different cell cultures both mycotoxins produce reactive oxygen species (ROS) with consequent oxidative damage (Hoehler et al., 1996; Schaaf et al., 2002; Stockmann-Juvala et al., 2004). ROS may directly damage DNA, or damage it through its products (Collins et al., 1996).

The oxidative DNA damage caused by ROS generated in the organism occurs constantly, and the kidney is a prominent site of intense oxidative processes in the body. The most easily formed DNA lesions induced by ROS are 8-oxo-7,8-dihydro-2′-deoxyguanosines (8-oxodG), but the background levels of altered purines are usually efficiently repaired. Certain compounds increase the level of the mutagenic 8-oxodG that plays an important role in carcinogenesis (Loft and Poulsen, 1996; Halliwell and Gutteridge, 1999). In the experiments, the level of 8-oxodG is used as a measure of oxidative

* Corresponding author. Tel.: +385 1 4673 188; fax: +385 1 4673 303.
E-mail address: adomijan@imi.hr (A.-M. Domijan).

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DNA damage. Various enzymatic methods based on the ability of DNA breaks to disrupt the physicochemical behavior of DNA molecule are used to detect oxidative DNA lesions (Collins et al., 2004). One of these is a modified version of the alkaline comet assay (single-cell gel electrophoresis). In general, the alkaline comet assay measures different types of DNA damage (double and single strand breaks, alkali labile sites, oxidative DNA base damage, crosslinking, DNA adducts). DNA breaks may relax DNA supercoiling and allow relaxed DNA loops to migrate under electrophoresis (Collins et al., 1997; ESCODD, 2003). Introducing formamido–pyrimidine–DNA–glycosylase (Fpg) in the assay on naked DNA after the lysis was performed, makes it possible to detect oxidative DNA damage. Fpg initiates the repair of oxidized bases by excising them and cutting the sugar-phosphate backbone of the DNA molecule. Thus additional strand breaks are induced at the location of oxidized base, causing DNA relaxation and migration (Collins et al., 1997; ESCODD, 2003).

The aim of this study was to find out whether DNA lesions caused by OTA and FB_1 were due to oxidative stress or some other mechanism. As we found both OTA and FB_1 simultaneously in maize in Croatia in our earlier investigation (Domijan et al., 2005), we believed that it was important to see whether the two mycotoxins had a synergistic effect in vivo. DNA damage was measured in the kidney cells of rats treated with OTA or FB_1 alone or with the combination of the two, using the standard alkaline comet assay and the Fpg-modified comet assay. The results were compared to see whether the combination of the two mycotoxins produced a synergistic DNA damaging effect.

2. Materials and methods

2.1. Animal study

Adult male Wistar rats (190 g of weight) were kept in macrolone cages. The animals were fed on a standard diet for laboratory rodents (Pliva, Zagreb, Croatia) and had free access to water. The rats were randomized in 12 groups of 6 animals.

OTA and FB_1 (Sigma, St. Louis, MO, USA) were dissolved in 51 mM $NaHCO_3$ and given orally once a day.

Three groups of animals were treated with OTA (5 ng/kg b.w., 0.05 mg/kg b.w., and 0.5 mg/kg b.w., respectively) for 15 days.

Three groups of animals were treated with FB_1 (200 ng/kg b.w., 0.05 mg/kg b.w., and 0.5 mg/kg b.w., respectively) for 5 days.

Three groups of animals were treated with OTA for 15 days and with FB_1 for the last 5 days of OTA treatment. The doses were 5 ng OTA/kg b.w. + 200 ng FB_1/kg b.w. for the first group, 5 ng OTA/kg b.w. + 0.05 mg FB_1/kg b.w. for the second group, and 0.05 mg OTA/kg b.w. + 0.05 mg FB_1/kg b.w. for the third group.

Control groups were given 10 ml/kg b.w. of solvent (51 mM $NaHCO_3$) or redistilled water for 15 days. Positive controls were given a single dose of ferric nitrilotriacetate (Fe-NTA, 15 mg Fe/kg b.w., i.p.), known to be a potent oxidant and renal carcinogen. This group of animals was sacrificed 4 h after treatment.

All other animals were sacrificed by cervical dislocation 24 h after the last dose. Kidneys were collected on ice, washed in saline, and immediately homogenized for 8-oxodG adduct analysis.

The principles of good laboratory animal care were followed throughout, as well as the Croatian Law for the Well-being of Animals. The Institute's Ethical Committee approved the study.

2.2. Comet assay and Fpg-modified comet assay

Tissue samples were homogenized in a chilled homogenization buffer (pH 7.5) containing 75 mM NaCl and 24 mM Na_2EDTA to obtain a 10% tissue solution. A Potter-type homogenizer was used, and kidney samples remained on ice during and after homogenization (Sasaki et al., 1997).

The analysis of 8-oxodG was performed using a Fpg FLARE™ assay kit (Trevigen Inc, Gaithersburg, USA). Within the kit the manufacturer provided all the reagents used. Slide preparations were performed according to the manufacturer's manual. A low melting point (LMP) agarose was melted and stabilized in water bath at 37 °C. For each animal, control or treated, 10 μl of cell homogenate was mixed with 300 μl of LMP agarose. Before use, the FLARE™ slides (provided with FLARE™ assay kit) were chilled at 2 °C for 30 min. The suspension (150 μl) was dropped on each of two slides. One of the two slides underwent Fpg treatment, and the other was treated with the provided buffer solution. Slides were kept in the dark at 2 °C for 20 min to improve the adherence of gels. Slides were then immersed in a pre-chilled lyses solution (provided with the FLARE™ assay kit) and kept in a refrigerator at 2 °C for 60 min. Followed the immersion in the FLARE™ buffer, three times for 15 min. After lysis treatment one slide for each animal was treated with 100 μl of Fpg enzyme per sample area (1:500 in REC dilution buffer). Enzyme dilution was prepared right before use. Sample areas of the other slide were treated with 100 μl of REC dilution buffer only. Slides were placed horizontally in humidity chamber at 37 °C for 45 min. All slides were then immersed in an alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) two times for 15 min. Followed electrophoresis in a pre-chilled alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1), on 1 V/cm for 20 min. The slides were then immersed in 70% ethanol for 5 min and air-dried.

Slides were stained with ethidium-bromide (20 μg/ml) for 10 min. Each slide was analyzed using the Leitz Orthoplan